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Possible carriers in mouse erythrocytes

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Early qualitative erythrocyte permeability studies (Gryns, 1896; Hedin, 1897) revealed the wide range of non-electrolyte permeability rates across a cell membrane composed of lipid molecules. Theoretically, water-soluble non-electrolytes such as glycerol, ethylene glycol and erythritol would penetrate less rapidly than lipid-soluble non-electrolytes. It was thought that the rate of penetration of water-soluble non-electrolytes was inversely proportional to their molecular size and the penetration of lipid-soluble non-electrolytes was directly proportional to their lipid-solubility.

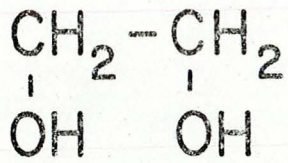
In the 1930's quantitative differences were observed in the rate of penetration of non-electrolytes which could not be explained on the basis of lipid-solubility or molecular size. Ulrich (1934) studied polyhydric alcohols and sugars in a number of species, and found that the 75% hemolysis time of mouse erythrocytes in an isotonic erythritol solution was a few minutes compared to eight hours in ox red cells. The inhibition of the penetration of glycerol into human and rat cells by trace amounts of copper was shown by Jacobs and Corson (1934). Jacobs, Glassman and Parpart (1935) related the rate of penetration and species differences using ethylene glycol and glycerol. (See Table 1) They showed that investigated species could be divided into two groups. The group consisting of rat, mouse, rabbit, man and guinea pig displayed a high permeability to glycerol with hemolysis times ranging from ten seconds to several minutes.

Table 1

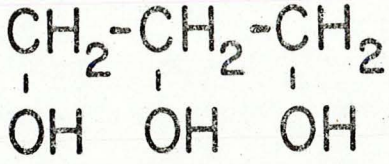
Chemical formulae of experimental non-electrolytes.

TABLE 1

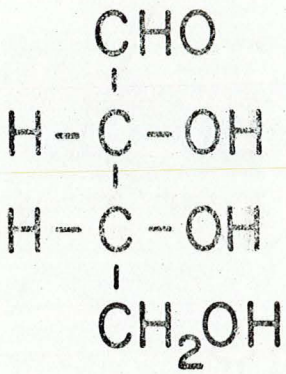
Ethylene Glycol



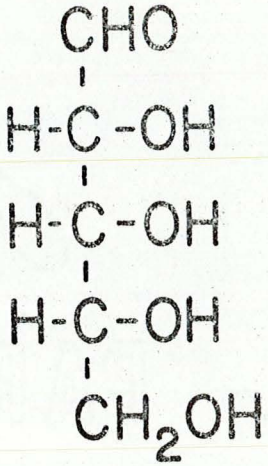
Glycerol



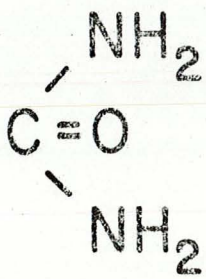
Erythritol



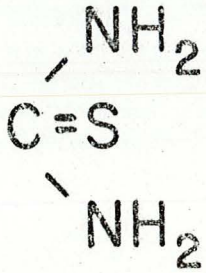
D-Ribose



Urea



Thiourea



The second group, cat, dog, horse, pig, sheep and ox had a much lower permeability to glycerol. In this group hemolysis times ranged from 15 to 20 minutes. Erythrocytes of the first group exhibited the pecularity that glycerol penetration was inhibited by traces of copper and was sensitive to changes in the pH of the solutions. The presence of copper and/or changes in pH had little or no effect on the permeability of glycerol in the cells of the second group.

The temperature coefficients (Q_{10}) of the first group were unusually low (less than two) while those of the second group high (greater than three). Such observations led Jacobs to suggest that the non-electrolytes, glycerol and ethylene glycol, might penetrate the erythrocytes of rat, mouse, rabbit, man and guinea pig by a mechanism other than simple diffusion.

The hypothesis that glycerol might be a case of "special" permeability was strengthened by the study of Jacobs and Parpart (1937) on the effect of n-butyl alcohol on the permeability of erythrocytes of several species. They found that n-butyl alcohol decreased glycerol permeability in man, rat, rabbit, guinea pig, ground hog and several birds. The presence of n-butyl alcohol increased glycerol permeability in the ox, pig, horse, dog and cat red cells. These two groups were essentially the same as outlined by Jacobs et al (1935) with respect to copper inhibition, sensitiveness to pH changes, and temperature coefficients.

Jacobs, Glassman and Parpart (1938) compared rat and mouse red cell permeabilities to erythritol, mannitol, glycerol and thiourea. They found that rat erythrocytes were more permeable

to glycerol than thiourea. The reverse was true for mouse erythrocytes. Mouse erythrocytes also displayed a high degree of permeability to erythritol whereas it penetrated rat red cell much more slowly.

The human-glycerol and human-glucose systems were extensively investigated by LeFevre (1948). His kinetic studies of volume changes in glucose-saline solutions indicated that glucose penetrated the erythrocytes by a mechanism other than simple diffusion. He further found that mercury, iodine and phlorizin depressed glucose penetration, which led him to suggest that the intermediation of a sulfhydral group on the membrane surface was involved in the passage of glycerol and glucose across the erythrocyte membrane.

Earlier investigations (Jacobs, 1931; Jacobs et al, 1935; Jacobs and Parpart, 1937) prompted Jacobs, Glassman and Parpart (1950) to examine the inter-class erythrocyte permeabilities of mammals, amphibians, fish, reptiles and birds. Urea, thiourea, glycerol and ethylene glycol isosmotic solutions were used. Their results, generally consistent within a given class, indicated an unusually high permeability constant for glycerol and ethylene glycol in bird erythrocytes and for urea in mammalian red cells.

Subsequent investigations concerning the transport of hexoses and glyercol (LeFevre, 1954; LeFevre and LeFevre, 1952; Widdas, 1951, 1952, 1954; Rosenberg and Wilbrandt, 1955; Stein and Danielli, 1956) showed that glucose and glycerol enter human red cells much more rapidly than could be accounted for by simple diffusion.

Competitive inhibition was observed between D-glucose, D-galactose, D-mannose, L-sorbose, D-fructose, D-xylose, L-arabinose, and meso-erythritol and penta-erythritol (LeFevre and Davies, 1951; Widdas, 1954; Bowyer and Widdas, 1955). Stein and Danielli (1956) have observed the inhibition of glycerol by the glycols and narcotics.

These studies have not only assisted in establishing the concept of facilitated diffusion involving a carrier mechanism but suggest that facilitated diffusion is a widespread process. LeFevre (1954) considers the simplest model to consist of (1) the formation of the substrate-carrier complex on the outside of the membrane, (2) the movement of the complex through the membrane, and (3) the uncoupling of the substance from the carrier upon arrival at the inside face of the membrane. The source of energy for the movement of the complex is thermal agitation. The equilibria reached are the same as those attained by simple diffusion. The rate of penetration may be expected not to be directly proportional to concentration but to reach a limiting (saturation) value as concentration is increased. LeFevre (1954) considers the dissociation rate of the complex the rate limiting factor.

Beginning in the 1960's, Hunter studied previously reported cases of "unusual" non-electrolyte permeabilities in several animals. He observed the effect of n-butyl alcohol on thiourea and glycerol penetration in human, rabbit, sheep and chicken erythrocytes in 1961. In each case where it was assumed that simple diffusion occurred (human, sheep, rabbit and chicken) n-butyl alcohol increased the thiourea permeability of the

respective erythrocytes. The glycerol permeability was decreased by n-butyl alcohol in those instances where glycerol was postulated to cross the membrane by a carrier mechanism. Hunter attributes the change in permeability to an alteration in the aqueous channels of the membrane. New channels are opened to increase permeability, and existing channels are closed to decrease permeability.

Subsequent studies (Hunter, George and Ospina, 1965; Ospina and Hunter, 1966) helped to distinguish between simple and facilitated diffusion systems using both n-butyl alcohol and tannic acid. These data suggested that carrier systems were found in the following: Urea--man, mouse and rabbit; Erythritol--mouse and possibly man; Ribose--mouse and man; Ethylene glycol--man and possibly mouse; Glycerol--man, mouse and rabbit.

Hunter (1970) following a suggestion of Jacobs et al, (1950) demonstrated that glycerol, erythritol and urea crossed the pigeon red cell membrane by a carrier mediated process. Competitive inhibition of urea by thiourea further suggested that the two shared the same carrier.

Reported instances of "special" or "unusual" permeabilities suggested the existence of a carrier meachanism for non-electrolyte penetration into mouse erythrocytes. The presence of carrier mechanisms in the erythrocytes of the mouse, Mus musculus, are established in this study. Saturation and competitive inhibition kinetics are used to calculate the half-saturation constants (o) for cases of facilitated transfer of the following water-soluble non-electrolytes: ethylene glycol, glycerol, erythritol, ribose, urea and thiourea.

These non-electrolytes are often used in permeability studies. They are non-toxic and their small size allows penetration of the erythrocyte membrane within a reasonable length of time. The first four non-electrolytes form a series of compounds of increasing number of carbons (two in ethylene glycol to five in ribose) and hydroxyl groups. Permeability investigations utilizing this family of penetrants show the effects of increasing size on penetration rate.

Urea and thiourea permeability studies illustrate the effect of substitution of a specific element on the penetration rate. (See Table 1)

METHODS AND MATERIALS

METHODS AND MATERIALS

Theoretical

Fick's First Law of Diffusion, equation (1), gives the relationship between solute rate of transport across a cell membrane and the concentration gradient, suggesting the simple uninhindered passive diffusion of solutes across a membrane.

$$\frac{dS}{dt} = - K (C-D) \qquad (1)$$

K represents the permeability constant. C is the external medium solute concentration. D is the solute concentration inside the cell. The rate of penetration (dS/dt) is thus dependent upon the concentration gradient between the outside and inside membrane faces.

Widdas (1951, 1952, 1954) adapted equation (1) to fit those instances where a carrier carrier mechanism was involved in the transfer of a penetrant across the cell membrane. In instances of facilitated diffusion, he postulated that the transfer rate is proportional to the difference between the fraction of carriers combined with penetrant on the outside and inside faces of the membrane. This relationship is represented by the following equation:

$$\frac{dS}{dt} = K\left\{\frac{C}{C+\phi} - \frac{S/V}{(S/V)+\phi}\right\}, \qquad (2)$$

where S is the amount of penetrating solute in the cell, C the external medium solute concentration, K the permeability constant, V the cell water volume, and ϕ the half-saturation constant of the carrier-penetrant complex.

LeFevre (1961) presented the integrated form of equation (2) as

$$k^{o}t=F^{o}(C,V)=(C+1)(C+\phi)\left\{(C+\phi+1)(V_{o}-V) + (C+\phi)\ln\frac{1-V_{o}}{1-V}\right\} \quad (3)$$

where k^{o} is the penetration constant, V_{o} the initial volume, V the calculated volume, C' the initial internal penetrant concentration, and C the external concentration.

Substitution of

$$V_{o}=\frac{1+C'}{1+C} \quad (4)$$

into equation (3) gives

$$k^{o}t=F^{o}(C,V)=(C+\phi)\left\{(C'+1)(C+\phi+1)-(C+1)(C+\phi+1)V+(C+1)(C+\phi)\cdot\ln\frac{C-C'}{(1-V)(C+1)}\right\} . \quad (5)$$

This basic equation (5) can be simplified for the two extreme cases where ϕ is relatively large with respect to S (the amount of penetrant inside the cell) and where ϕ is small with respect to S. Integration of these two extreme limiting conditions gives equation (5) the following forms:

when ϕ is large (diffusion-type)

$$kt=F'(C,V)=C'+1-(1+C)V+(1+C)\ln\frac{C-C'}{(1-V)(1+C)} , \quad (6)$$

when ϕ is small (near-saturation carrier type)

$$k't=F'(C,V)=C(1+C)\left\{C'+1-(1+C)V+C\cdot\ln\frac{C-C'}{(1-V)(1+C)}\right\} . \quad (7)$$

In both instances k is the penetration constant, V the cell water volume, t the time, C' the initial internal penetrant concentration, and C the external solute concentration. In the first case, equation (6), the concentration gradient across the membrane is

rate limiting, while in the second approximation, equation (7), the saturation of the carrier is rate limiting.

Experimentally the concentration of the penetrant with which the cells have previously been equilibrated (C') and the concentration of the non-electrolyte to be added to the solution (C) are known for each addition of non-electrolyte solute. The substitution of these values of C' and C and selected volumes (V) reduces LeFevre's equation (5) to the form

$$k^{o}t=F^{o}(C,V)= A + B\phi + C\phi^{2} . \qquad (8)$$

These values of A, B, and C for the selected volumes can be tabulated. (See Tables 2, 3, 4, 5) As the value of ϕ approaches zero, the B and C terms become neglible with respect to the A term. When the value of ϕ approaches infinity, the A and B terms are neglible with respect to the C term.

The times required to reach the selected volumes used to calculate the table are measured on the recorded swelling curves. Plotting the experimental times against the table values of F(C,V) and F'(C,V) gives graphs which describe the type of kinetics involved in the penetration of the non-electrolyte across the erythrocyte membrane.

Diffusion-type kinetics is suggested where a plot of the experimental times against the appropriate values of F(C,V) gives a single straight line. The slope of this line represents the penetration constant k.

Keeping in mind that F'(C,V) was derived for the situation where the carrier was nearly saturated (ϕ small with respect to the solute concentration S), then a single straight line in a

plot of experimental times against the values of the function F'(C,V) indicates that the kinetics are those of a near-saturated carrier.

If both the F(C,V) and F'(C,V) plots give a family of straight lines with symbols reversed (cf. Figures 3 and 5) then a plot of LeFevre's $F^{o}(C,V)$ using an appropriate estimate of ϕ should give a single straight line. Thus a plot of $F^{o}(C,V)$ against experimental times provided a method of approximating the value of the half-saturation constant (ϕ).

Widdas (1952) extended his kinetic analysis to include competitive inhibition on theoretical grounds. In a later investigation (Widdas, 1954) he showed that the transfer of sorbose in the presence of glucose could be described by:

$$\frac{dS_s}{dt} = K\left\{\frac{C_s}{C_s+\phi_s+\frac{\phi_s}{\phi_g}C_g} - \frac{C_s'}{C_s+\phi_s+\frac{\phi_s}{\phi_g}C_g'}\right\}, \qquad (9)$$

where C_s is the sorbose concentration of the outside medium, C_g the glucose (inhibiting penetrant) outside medium concentration, and C_g' and C_s' refer to the concentrations inside the cell. The half-saturation constants of the glucose- and sorbose-carrier complexes are ϕ_g and ϕ_s respectively.

Assuming that ϕ_s is large relative to the other concentrations equation (9) can be written

$$\frac{dS_s}{dt} = K\left\{\frac{C_s}{\phi_s+\frac{\phi_s}{\phi_g}C_g} - \frac{C_s'}{\phi_s+\frac{\phi_s}{\phi_g}C_g'}\right\}. \qquad (10)$$

By making certain simplifying assumptions, equation (10) reduces to

$$\frac{1}{k} = \frac{\phi_s}{K} + \frac{\phi_s}{\phi_g} \cdot \frac{\phi_g}{K} \quad , \qquad (11)$$

where the reciprocal of the apparent penetration constant k varies linearly with the glucose (inhibitor) concentration. From a plot of 1/k (ordinate) against C_g (abcissa) a value of ϕ_g can be obtained by dividing the y-intercept by the slope. (See Figure 16)

$$\frac{\text{Intercept } (C_g=0)}{\text{slope}} = \phi_g \qquad (12)$$

This method of determining the value of the half-saturation constant is useful if the ϕ cannot be approximated using saturation kinetic studies. In other cases, it can be used as verification of half-saturation values determined through saturation studies.

Experimental

Mouse (Mus musculus) erythrocytes were used for all experiments. Blood was obtained by decapitation and collected in 10 ml of heparinized 0.9% NaCl buffered to a pH of 7.5 (6.05 g Tris buffer + 3.45 ml concentrated HCL per liter). The cells were washed three times in 20 ml of the buffered saline solution by centrifugation at low speeds for 60 to 90 seconds. After each centrifugation the supernatant and buffy layer were removed by aspiration. All washed, concentrated cells were used within four hours of preparation. All experimental solutions were prepared using the buffered 0.9% NaCl solution. The pH was measured with a Corning Model 7 pH meter.

Measurements of volume changes of the cells were made using a densimeter technique which was developed by Ørskov (1935) and modified by Hunter (1960, 1970). The densimeter chamber was surrounded laterally and below by a water jacket. A pump circulated water from a constant temperature bath through the chamber jacket for temperature control. A light beam passed through the densimeter chamber and surrounding water jacket falling on a photoelectric cell cathode. The shrinking or swelling of the suspended red cells resulted in decreases or increases in levels of light scattering respectively. These cell volume changes resulted from water movement out of or into the cells as non-electrolyte was added to the cell suspension.

The photoelectric cell cathode translated the level of light scattering into a D-C current. The current was then amplified by a D-C amplifier and transmitted to an Esterline Angus pen recorder which recorded the resultant curves. The sensitivity of the system was altered by changing the concentration of the blood, the settings of the D-C amplifier, and the speeds of the pen recorder.

Generally, an aliquot (0.05-0.2 ml) of the concentrated, washed cells was suspended in and allowed to come to equilibrium with a known volume of the buffered isotonic (0.9%) saline solution contained in the densimeter chamber. The suspension was stirred during all experimental runs by a motor-driven glass stirring rod. Prior to adding the non-electrolyte, the recorder was turned on to record the series of swelling curves. A known volume of penetrating non-electrolyte was then rapidly added by

syringe to the suspension. The volume of cell water exiting from the cells with the addition of the non-electrolyte was small compared to the total suspension volume; hence, osmotic pressure changes were due to the addition of the non-electrolyte and not appreciably altered by the addition of the small amount of cell water to the suspension.

Once equilibrium was established, additions of non-electrolyte were made. The concentration of penetrant was known before and after each addition. All concentrations were calculated in terms of isotonic values (isotones). An isotonic solution contains one isotone and in such a solution, a cell has a volume of one. (A 0.3 M solution of a non-electrolyte is isotonic with mammalian blood.)

The initial downward deflection of the recorder pen after an addition of non-electrolyte results from the rapid exit of water out of the cells since the added solution of penetrant is hypertonic to the cells. The cells shrink and less light is transmitted through the cell suspension. With successive additions of equal volumes of penetrant solutions, the concentration gradient across the cell membrane decreases and less and less water exits from the cells with each subsequent addition of penetrant. This means that the minimum volume is larger with each addition; consequently, the total deflection of the pen decreases.

Tables of $F(C,V)$, $F'(C,V)$ and $F^{o}(C,V)$ functions for selected volumes provided the theoretical values for my study. (See Tables 2, 3, 4, 5.) In the 0.8 M system, Table 2, 3.0 ml of a 0.9% NaCl-blood suspension were equilibrated in 3.0 ml of

TABLE 2

Calculated values of $F(C,V)$, $F'(C,V)$ and $F^{o}(C,V)$ for the following system. 3.0 ml of a cell-0.9% NaCl suspension are equilibrated in 3.0 ml of 0.5 M penetrant in 0.9% NaCl. After equilibrium has been reached, six successive additions of 0.8 M penetrant in 0.9% NaCl are made and recorded. Time is allowed for each addition of penetrant to establish equilibrium before the next addition is made.

TABLE 2

Initial conc. of penetrant (isotonic units) C'	Final conc. of penetrant (isotonic units) C	Relative Volume of Cell Water V	$F^{o}(C,V)$ A As $\phi \longrightarrow 0; F'(C,V)$	$F^{o}(C,V)$ B	$F^{o}(C,V)$ C As $\phi \longrightarrow \infty; F(C,V)$
0.833	1.095	0.90	0.441	0.858	0.416
		0.92	0.905	1.748	0.841
		0.95	1.942	3.705	1.763
		0.98	4.100	7.708	3.620
1.095	1.292	0.93	0.684	1.095	0.438
		0.95	1.828	2.913	1.159
		0.97	3.647	5.774	2.284
		0.98	5.041	7.954	3.137
1.292	1.443	0.95	0.977	1.383	0.489
		0.96	2.026	2.861	1.010
		0.98	5.380	7.558	2.654
		0.99	8.820	12.351	4.323
1.443	1.567	0.96	1.103	1.429	0.463
		0.98	3.255	5.510	2.191
		0.99	9.532	12.264	3.945
1.567	1.667	0.98	4.451	5.386	1.630
		0.99	9.469	11.434	3.451
1.667	1.750	0.98	3.331	3.835	1.104
		0.99	9.038	10.384	2.982

TABLE 3

Calculated values of F(C,V), F'(C,V) and $F^{o}(C,V)$ for the following system. Successive 0.1, 0.2, 0.4, 0.6, and 0.8 ml additions of 1 M non-electrolyte in 0.9% NaCl added to 9.9 ml of cell-0.9% NaCl suspension. Time is allowed following each addition of penetrant for equilibrium to be reached before next addition.

TABLE 3

Initial conc. of penetrant (isotonic units) C'	Final conc. of penetrant (isotonic units) C	Relative Volume of Cell Water V	$F^{o}(C,V)$ A As $\phi \longrightarrow 0; F'(C,V)$	$F^{o}(C,V)$ B As $\phi \longrightarrow \infty; F(C,V)$	
0	0.033	0.98	0.0001	0.020	0.474
		0.99	0.0005	0.055	1.175
0.033	0.098	0.96	0.0019	0.059	0.409
		0.97	0.0037	0.108	0.712
		0.98	0.0069	0.183	1.148
		0.99	0.0128	0.317	1.898
0.098	0.220	0.92	0.0068	0.086	0.248
		0.94	0.0174	0.206	0.577
		0.96	0.0345	0.387	1.045
		0.98	0.0689	0.723	1.865
0.220	0.387	0.92	0.0553	0.342	0.515
		0.94	0.0998	0.600	0.884
		0.96	0.1687	0.984	1.416
		0.98	0.2984	1.681	2.351
0.387	0.583	0.92	0.1717	0.659	0.624
		0.94	0.2963	1.117	1.044
		0.96	0.4855	1.800	1.657
		0.98	0.8298	3.011	2.722

TABLE 4

Calculated values of $F(C,V)$, $F'(C,V)$ and $F^{o}(C,V)$ for the following system. 0.2 ml of cells in 0.9% NaCl are equilibrated in 7.8 ml of a 0.6 M non-electrolyte in 0.9% NaCl solution. This is followed by four additions of 1 ml each of a 2 M non-electrolyte in 0.9% NaCl solution. Time is allowed following each addition of penetrant for equilibrium to be established before the next addition.

TABLE 4

Initial conc. of penetrant (isotonic units) C'	Final conc. of penetrant (isotonic units) C	Relative Volume of Cell Water V	$F^{o}(C,V)$ A As $\phi \longrightarrow 0; F'(C,V)$	B	C As $\phi \longrightarrow \infty; F(C,V)$
1.95	2.47	0.90	7.11	5.94	1.24
		0.92	11.16	9.29	1.93
		0.94	16.72	13.86	2.87
		0.96	24.72	20.41	4.21
2.47	2.89	0.92	8.53	6.01	1.06
		0.94	17.02	11.96	2.10
		0.96	29.28	20.53	3.60
		0.98	50.92	35.57	6.21
2.89	3.24	0.94	12.99	8.12	1.27
		0.95	20.35	12.70	1.98
		0.96	29.68	18.52	2.89
		0.98	63.12	39.21	6.09
3.24	3.52	0.96	23.34	13.35	1.91
		0.97	38.16	21.86	3.13
		0.98	60.30	34.45	4.92

TABLE 5

Calculated values of $F(C,V)$, $F'(C,V)$ and $F^{o}(C,V)$ for the following system. Cells are added to 8.5 ml of 0.9% NaCl. Eight additions of 0.5 ml each of 8.1 M non-electrolyte in 0.9% NaCl are made. Time is allowed following each addition of penetrant for equilibrium to be reached before next addition.

TABLE 5

Initial conc. of penetrant (isotonic units) C'	Final conc. of penetrant (isotonic units) C	Relative Volume of Cell Water V	$F^{o}(C,V)$ A As $\phi \longrightarrow 0$; $F'(C,V)$	B	C As $\phi \longrightarrow \infty$; $F(C,V)$
0	1.50	0.90	5.39	8.44	3.23
		0.92	6.44	9.89	3.73
		0.94	7.88	11.85	4.40
		0.96	9.99	14.73	5.38
1.50	2.84	0.90	28.20	20.81	3.83
		0.92	34.25	25.15	4.61
		0.94	42.40	30.98	5.65
		0.96	54.27	39.39	7.14
2.84	4.05	0.90	57.71	29.19	3.69
		0.92	74.64	37.67	4.75
		0.95	110.20	55.36	6.95
		0.97	150.62	75.42	9.44
4.05	5.14	0.90	77.61	30.67	3.03
		0.92	110.56	43.61	4.30
		0.95	180.83	71.16	7.00
		0.97	260.19	102.12	10.02
5.14	6.14	0.90	77.92	25.65	2.11
		0.92	131.95	43.41	3.57
		0.95	249.04	81.76	6.71
		0.98	487.33	93.25	13.06
6.14	7.04	0.90	39.64	11.33	0.81
		0.92	119.40	34.14	2.44
		0.95	293.00	83.72	5.98
		0.98	643.53	183.56	13.09
7.04	7.88	0.92	82.50	21.03	1.34
		0.94	230.57	58.81	3.75
		0.96	439.78	112.15	7.15
		0.98	809.99	206.25	13.13
7.88	8.64	0.94	179.19	41.65	2.42
		0.96	456.71	106.17	6.17
		0.98	939.34	218.02	12.65

0.5 M penetrant in 0.9% NaCl. After equilibrium had been reached, six successive additions of 0.8 M penetrant in 0.9% NaCl were made and recorded. Time was allowed for each penetrant addition to establish equilibrium before the next addition was made. The final penetrant concentration was 1.75 isotones (Hunter, unpublished data).

Table 3 gives a 1 M system developed by Hunter (1968). Successive additions (0.1, 0.2, 0.4, 0.6, 0.8 ml) of 1 M penetrant in 0.9% NaCl were added to 9.9 ml of the 0.9% NaCl-blood suspension with a final penetrant concentration of 0.58 isotones.

Four 1 ml additions of 2 M penetrant in 0.9% NaCl were added to 8 ml of a 0.6 M penetrant-blood suspension in the 0.9% NaCl in the 2 M system, Table 4. The final concentration in this system was 3.52 isotones (Hunter, unpublished data).

Eight successive 0.5 ml additions of 8.1 M penetrant in 0.9% NaCl were added to 8.5 ml of the 0.9% NaCl-blood suspension in the 8.1 M system, Table 5. In this system, the final penetrant concentration was 8.64 isotones (Hunter, 1970).

For competitive inhibition studies, a series of swelling curves was obtained. The control swelling curves were made using only one penetrant, the non-electrolyte expected to be inhibited. A subsequent series of swelling curves was made with the cell suspension being equilibrated in known increasing concentrations of a possible inhibiting non-electrolyte before the additions of the first penetrant were added. (See Figures 14 and 15) A plot of the reciprocals of the penetration constants (k) of the inhibited penetrant against the isotonic concentration of the

inhibiting penetrant gives a straight line with an ordinate intercept ($C_{\text{Inhibited substance}}=0$) and a slope of m (Figure 16). Using Widdas' equation (12) the half-saturation constant (ϕ) of the inhibiting non-electrolyte can be obtained.

At low concentrations of penetrating non-electrolyte, the response of the apparatus was linear. However, at higher concentrations (8.1 M system in particular) the response was not linear. Consequently, a calibration curve, Figure 2, was constructed relating deflection to theoretical volume in each case of non-linear response.

RESULTS

RESULTS

Figure 1 shows a typical series of swelling curves as equal additions of 8.1 M ethylene glycol in 0.9% NaCl were made to the cell suspension. In the 8.1 M system the concentration gradient across the cell membrane decreases with each addition. However, the decrease in initial slope with each succeeding addition is greater than would be expected if the ethylene glycol enters the cells by simple diffusion. Figure 2 gives a typical calibration curve used to establish the relationship between volume change and the total deflection. Selected volumes in the range of 0.9-1.0 were used in the analysis of the data.

SATURATION STUDIES

Glycerol

Studies conducted at 10°C using a 1 M glycerol in 0.9% NaCl solution gave no indication of saturation. Experimental times plotted against F(C,V) gave a single straight line and the same data when plotted against F'(C,V) gave a family of curves. Figures 3, 4 and 5 show data obtained from 8.1 M glycerol studies at 35°C. The F(C,V) plot, Figure 3, and the F'(C,V) plot, Figure 5, are families of straight lines with symbols reversed. Such a condition suggests that neither simple diffusion nor near-saturation kinetics applies and that an intermediate value of ϕ between zero and infinity would describe the kinetics of the system. The data fall on a single straight line when plotted against $F^{\circ}(C,V)$ with ϕ equal to five (Figure 4).

Ethylene Glycol

As with glycerol, additions of 8.1 M ethylene glycol in

0.9% NaCl at 35°C appeared to alter the cells' permeability to the extent that results were inconsistent at this concentration. Studies were then made using a 2 M ethylene glycol system at 10°C. Use of calibration curves was made as described above. Data from the 2 M system are plotted against F(C,V) in Figure 6, F'(C,V) in Figure 7, and $F^{o}(C,V)$ in Figure 8. The fact that the data obtained from the third and fourth additions fall on the same straight line in Figure 7 suggests near-saturation kinetics. The marked decrease in the initial slope of each succeeding curve is suggestive of saturation. The ethylene glycol concentration after the third addition was 3.24 isotones. Using a value of $\phi_{\text{ethylene glycol}}$ equal to 2, gives a close fit to a single straight line (Figure 8). An $F^{o}(C,V)$ plot with a $\phi_{\text{ethylene glycol}}$ of three gave a straight line with a slightly wider spread of points than that shown in Figure 8.

Erythritol

Data obtained with 2 M erythritol in 0.9% NaCl at 35°C are plotted against F(C,V) in Figure 9 and F'(C,V) in Figure 11. Figures 9 and 11 suggest diffusion-type kinetics at this concentration. Erythritol solutions of higher concentration were not used due to its low solubility in water.

Ribose

A 1 M ribose in 0.9% NaCl system was studied at 35°C. A plot of experimental times against values of F(C,V) and F'(C,V) gave results similar to those of erythritol (cf. Figures 9 and 11). These data suggested that ribose penetrated the cell by simple diffusion at the concentration of 0.58 isotones attained with

the 1 M ribose system.

Urea

Initially a 1 M urea in 0.9% NaCl solution was studied at 13° and 35°C. The effect of temperature on the apparent penetration rate was small. Due to the rapid rate of penetration of urea into the cells, additional studies were made at 10°C using an 8.1 M urea in 0.9% NaCl system. Calibration curves were constructed for each series of swelling curves as previously described. Data obtained with the 8.1 M urea system plotted against F(C,V) and F'(C,V) can be seen in Figures 12 and 13 respectively. These data suggest that urea penetrates the cell membrane by simple diffusion at the concentration reached by the 8.1 M system (8.64 isotones).

Thiourea

Studies using thiourea were conducted at 10°C. Due to the low temperature and low water solubility of thiourea, initial studies were made with an 0.8 M thiourea in 0.9% NaCl solution. Plots of experimental times against F(C,V) and F'(C,V) were similar to those of erythritol except that the rate of thiourea penetration was much more rapid than that of erythritol. Studies were also made at 35°C using a 1 M thiourea system. A plot of these data against values of F(C,V) and F'(C,V) gave results similar to the data obtained at 10°C.

COMPETITIVE INHIBITION STUDIES

Glycerol-Ethylene Glycol

Figure 15 (a) shows a typical series of 1 M glycerol swelling curves whereas Figure 15 (b) and (c) show the inhibition of glycerol penetration in the presence of 1.08 and 1.10 isotones of 1 M ethylene glycol, respectively. In Figure 14 the average times of inhibition studies are plotted against F(C,V). The rate of penetration of glycerol was progressively reduced in the presence of increasing concentrations of ethylene glycol. The apparent penetration constant of glycerol was reduced to a third its initial rate. Figure 16 shows the reciprocal of the apparent penetration constant of glycerol plotted against the ethylene glycol concentration in isotones for the 0.6 ml addition of the 1 M system. By dividing the intercept by the slope, an experimental value of the half-saturation constant (ϕ) of ethylene glycol was found to be 0.7 isotones. Similar calculations based on the 0.8 ml addition yielded a o value of 0.9 isotones.

Ribose-Erythritol

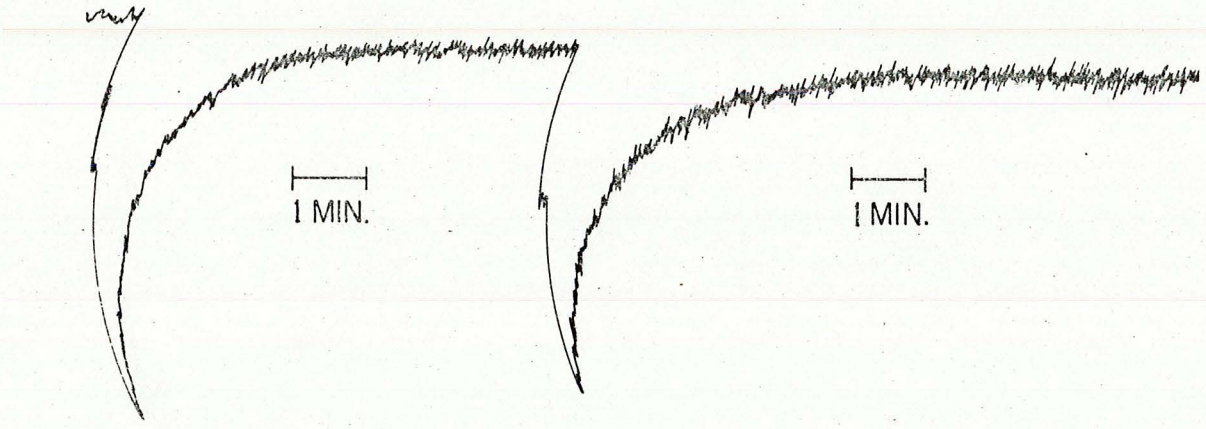
An F(C,V) plot of 1 M ribose entry times in the presence of increasing concentrations of 0.6 M erythritol was similar to that obtained in glycerol-ethylene glycol inhibition studies. The initial apparent penetration constant of 0.30 was reduced to 0.20 in the presence of 3 ml of 0.6 M erythritol (0.94 isotones). A plot of the reciprocals of the apparent penetration constants (1/k) against the erythritol concentrations in isotones yielded a ϕ value of 1.7 isotones for erythritol for the 0.6 ml

addition. Data from the 0.4 ml addition gave a ϕ value of 3.2 isotones for erythritol (Figure 17).

Urea-Thiourea

Initial studies in which urea was the inhibitor and thiourea the penetrating substance proved negative. Subsequent studies at 10°C were made equilibrating the cells in increasing concentrations of 0.8 M thiourea in 0.9% NaCl and then recording the resulting swelling curves as 1 M urea in 0.9% NaCl was added. An average of experimental times plotted against F(C,V) values is given in Figure 18. The apparent penetration constant for urea was reduced with successive additions of 0.8 M thiourea to one-half it's original value in Figure 18. On the basis of Figure 19, a ϕ with a value of 0.8 isotones was calculated for thiourea. Similar calculations were made on data obtained from a 0.6 ml addition F(C,V) plot giving a value of 0.9 isotones for thiourea.

FIGURE 1. Swelling of mouse erythrocytes following four successive additions of 0.5 ml of 8.1 M ethylene glycol in 0.9% NaCl. After each addition, position of the pen was moved downward by changing the setting on the amplifier. Temperature = 35°C.



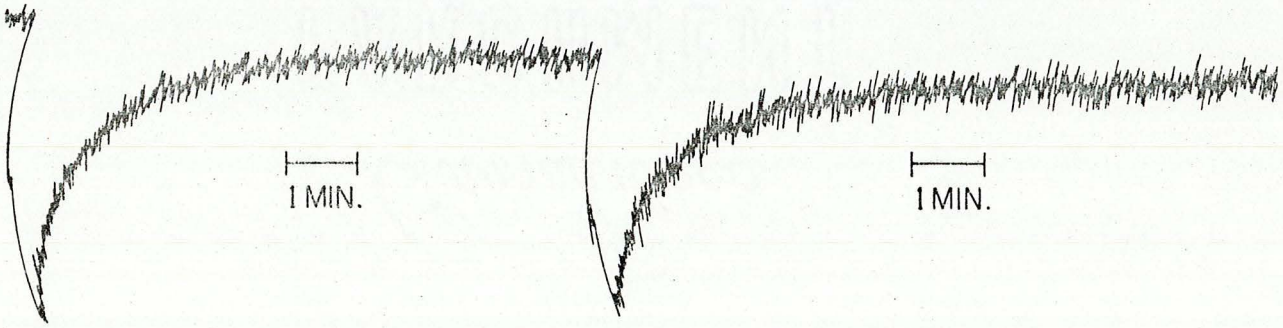


Fig. I

FIGURE 2. Calibration curve relating volume change to deflection of pen recorder. Abcissa: calculated minimum volume (V_o). Ordinate: total deflection in millimeters as measured on experimental curves. Eight 0.5 ml additions of 8.1 M ethylene glycol in 0.9% NaCl. Temperature = 10°C.

FIGURE 3. Average values of times for mouse erythrocytes to reach specified volumes as measured from experimental records, plotted against calculated values of F(C,V). There were eight successive additions of 0.5 ml of 8.1 M glycerol in 0.9% NaCl. Data from first four additions not included. △ :C'=5.24, C=6.14; ▽ :C'=6.14, C=7.04; ■ :C'=7.04, C=7.88; ▲ :C'=7.88, C=8.64 isotones. Symbols have same significance in Figures 4, 5, 12 and 13. Temperature = 35°C.

FIGURE 4. Same data as in Figure 3 plotted against values of $F^{o}(C,V)$, ϕ = 5 isotones.

FIGURE 5. Same data as in Figure 3 plotted against values of F'(C,V).

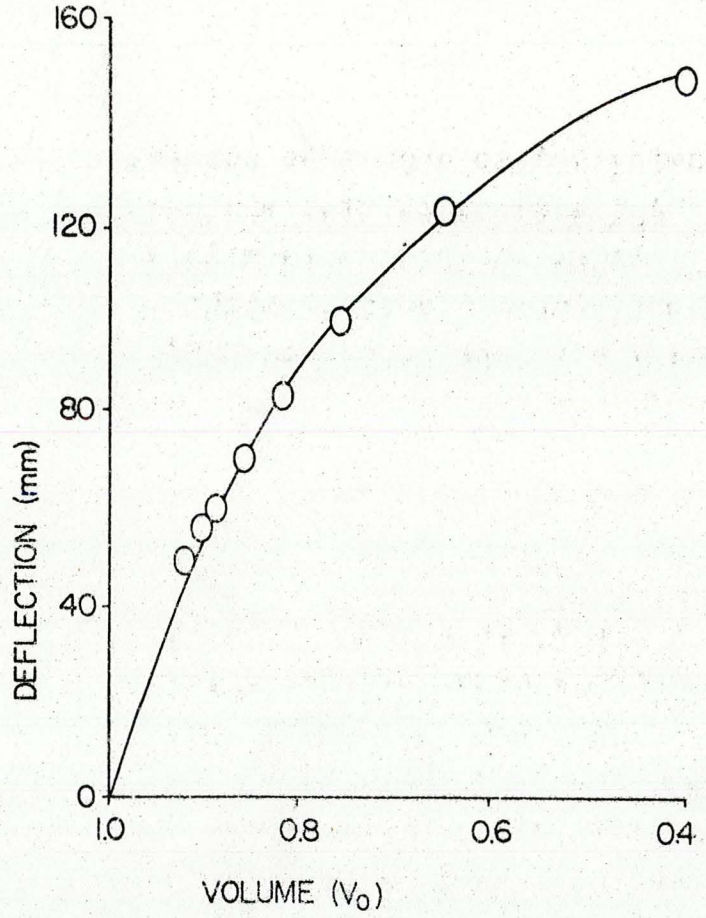


Fig. 2

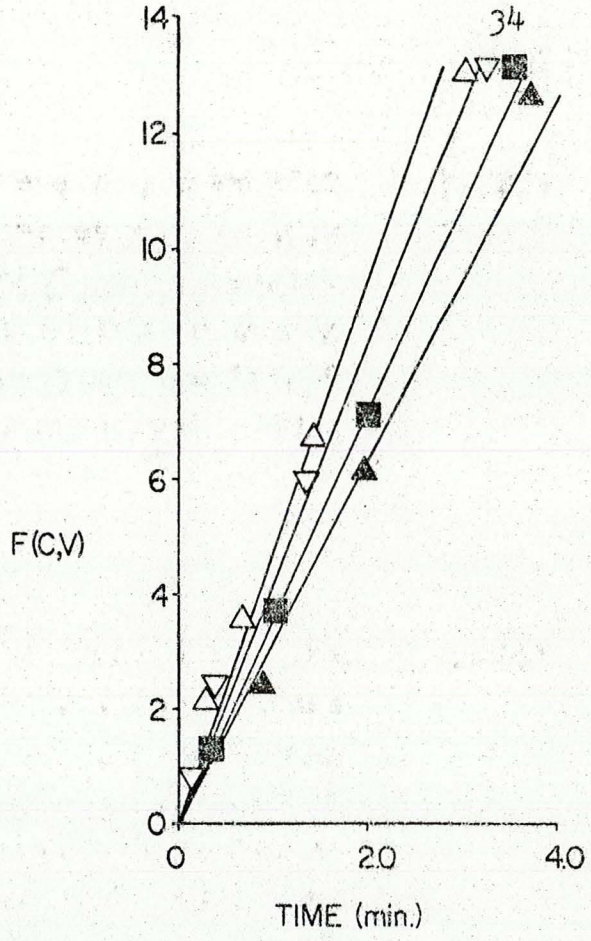


Fig. 3

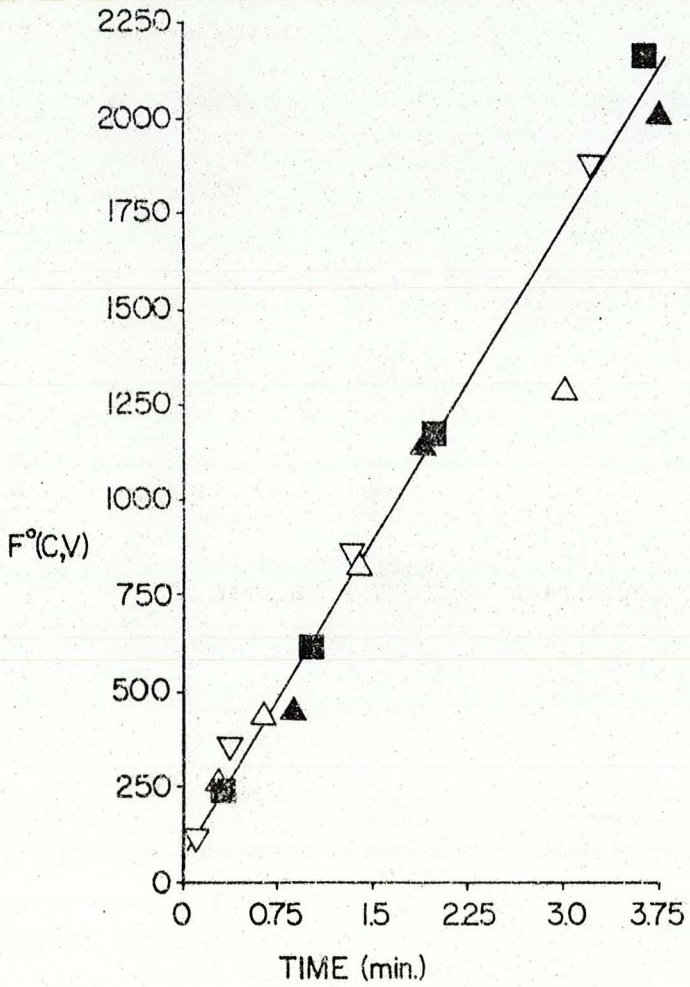


Fig. 4

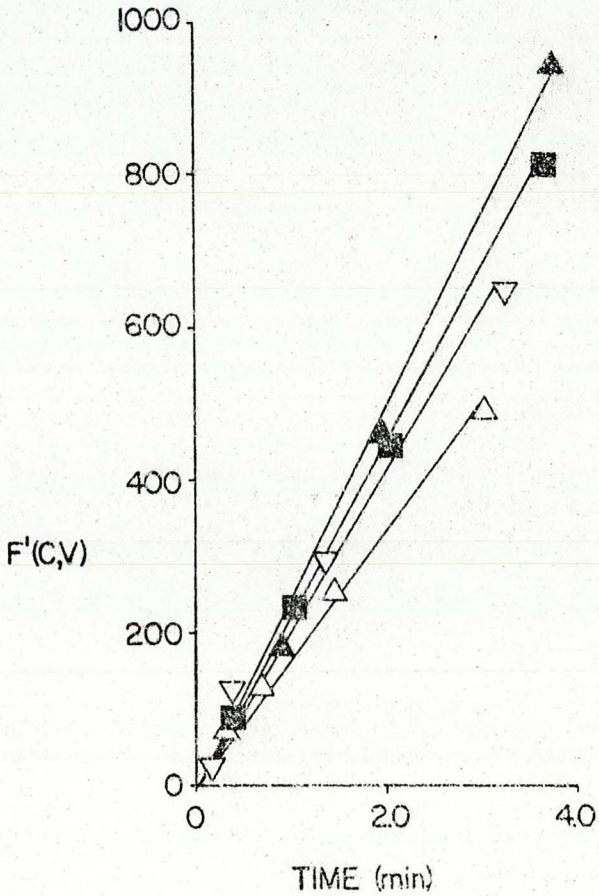


Fig. 5

FIGURE 6. Average values of times for mouse erythrocytes to reach specific volumes as measured from experimental records, plotted against calculated values of F(C,V). Cells were first equilibrated with 1.95 isotones of ethylene glycol, then four successive additions of 1 ml each of 2 M ethylene glycol in 0.9% NaCl were made. ● :C'=1.95, C=2.47; × :C'=2.47, C=2.89; ○ :C'=2.89, C=3.24; □ :C'=3.24, C=3.52 isotones. Symbols have same significance in Figures 6, 7, 8, 9, and 11. Temperature=10°C.

FIGURE 7. Same data as in Fig. 6 plotted against values of F'(C,V).

FIGURE 8. Same data as in Fig. 6 plotted against values of $F^{o}(C,V)$, ϕ=2 isotones.

FIGURE 9. Average values of times for mouse erythrocytes to reach specific volumes as measured from experimental records, plotted against calculated values of F(C,V). Cells were first equilibrated with 1.95 isotones of erythritol, then four successive additions of 1 ml each of 2 M erythritol in 0.9% NaCl were made. Temperature=35°C. Symbols have same significance as in Fig. 6.

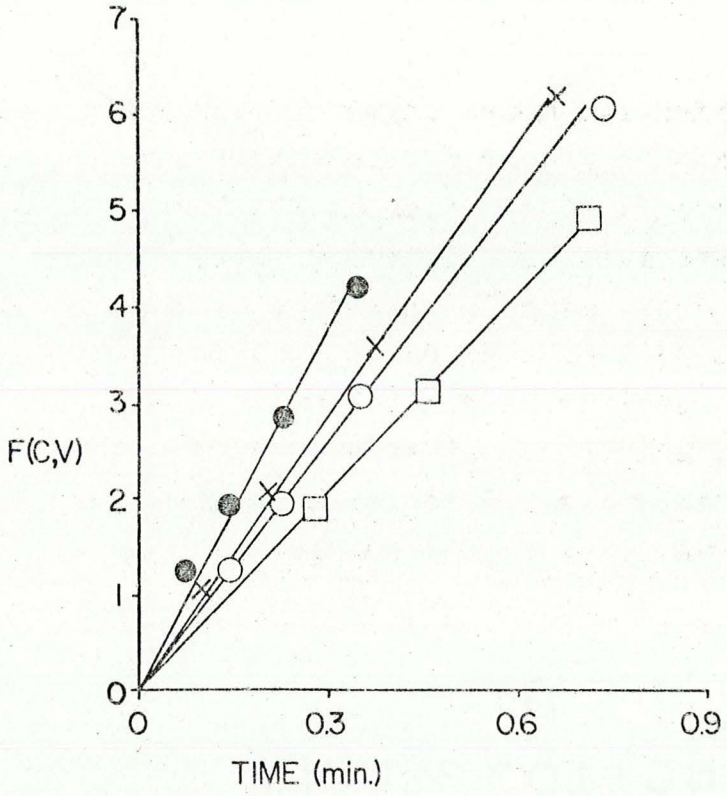


Fig. 6

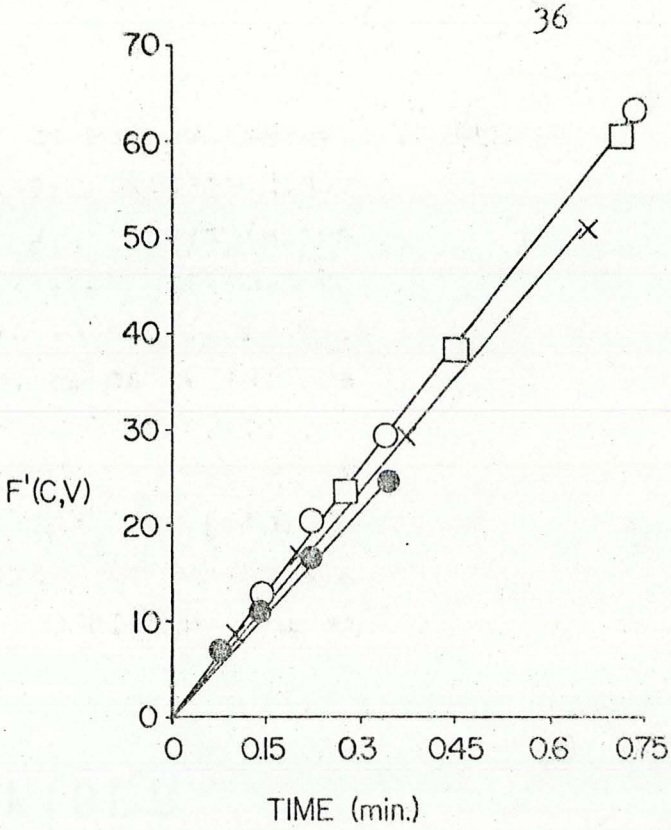


Fig. 7

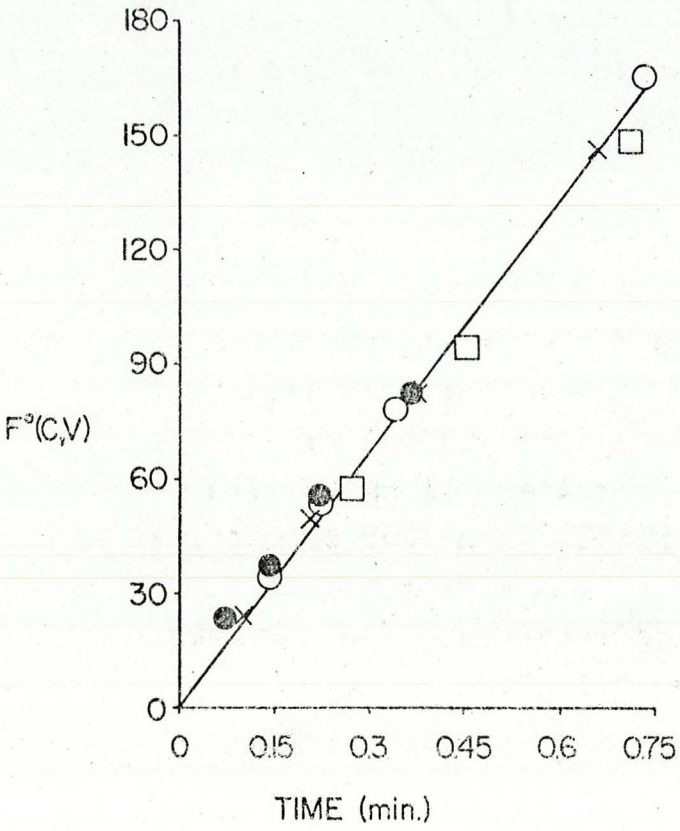


Fig. 8

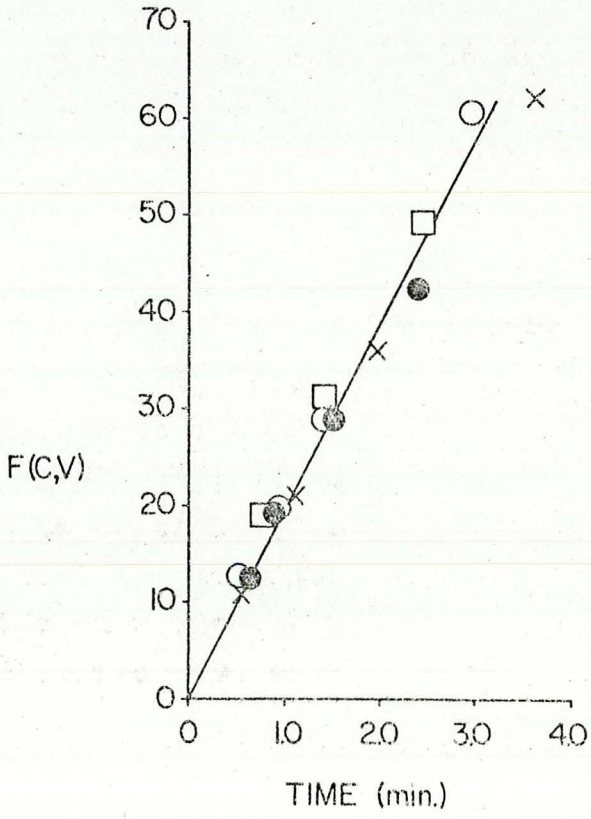
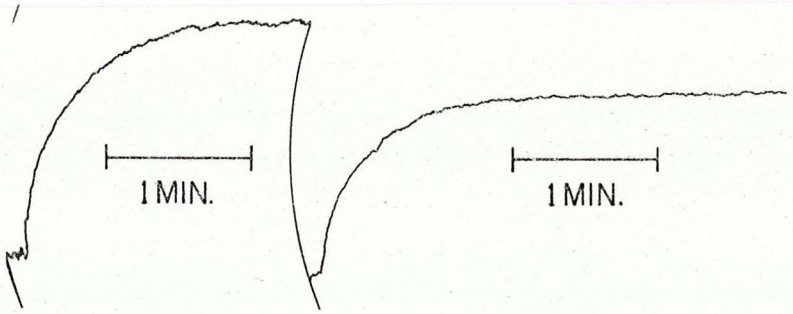


Fig. 9

FIGURE 10. Demonstration of Saturation. Note the decrease in the initial slope of each succeeding curve of mouse erythrocytes previously equilibrated in 0.6 M ethylene glycol. Four successive additions of 1 ml each of 2 M ethylene glycol in 0.9% NaCl were recorded. After each addition, position of pen was moved downward by changing the setting on the amplifier. Temperature=10°C.



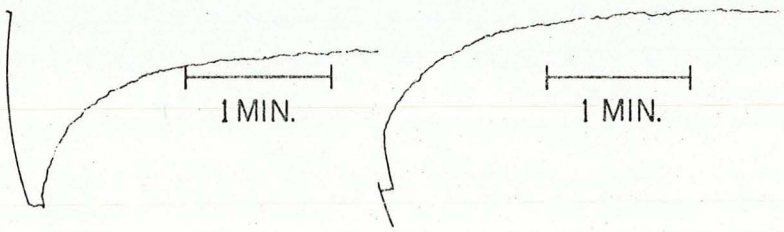


Fig. 10

FIGURE 11. Same data as in Figure 9 plotted against values of F'(C,V).

FIGURE 12. Average values of times for mouse erythroyctes to reach specified volumes as measured from experimental records, plotted against calculated values of F(C,V). There were eight successive additions of 0.5 ml of 8.1 M urea in 0.9% NaCl. Data from the first four additions were not included. Symbols have same significance as in Figure 3. Temperature = 10°C.

FIGURE 13. Same data as in Figure 12 plotted against values of F'(C,V).

FIGURE 14. Demonstration of competitive inhibition in mouse erythrocytes previously equilibrated in increasing concentrations of ethylene glycol. Using the 1 M system average values of times for 0.6 ml addition to reach specified volumes as measured from experimental records plotted against values of F(C,V). Glycerol apparent penetration constant (k) varies with different concentrations of ethylene glycol in isotones (C_{eg}). Temperature = 35°C.

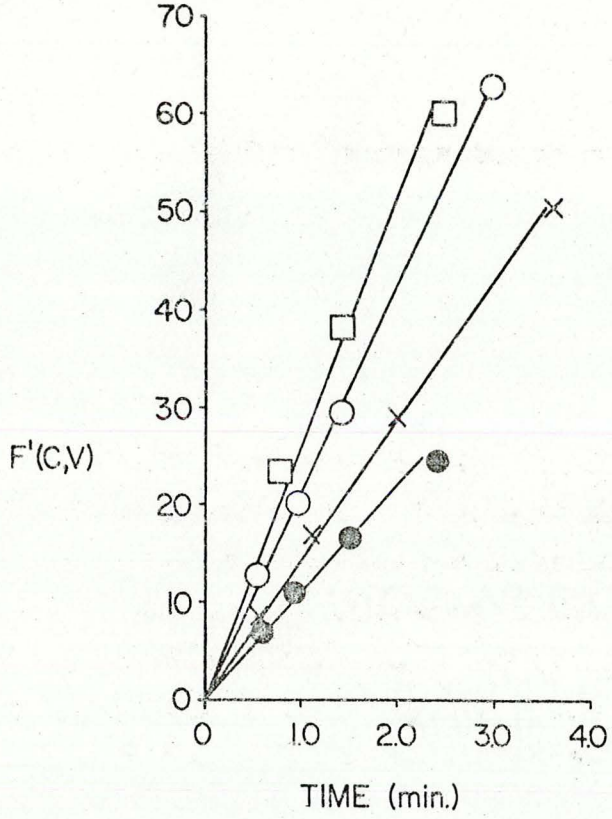


Fig. 11

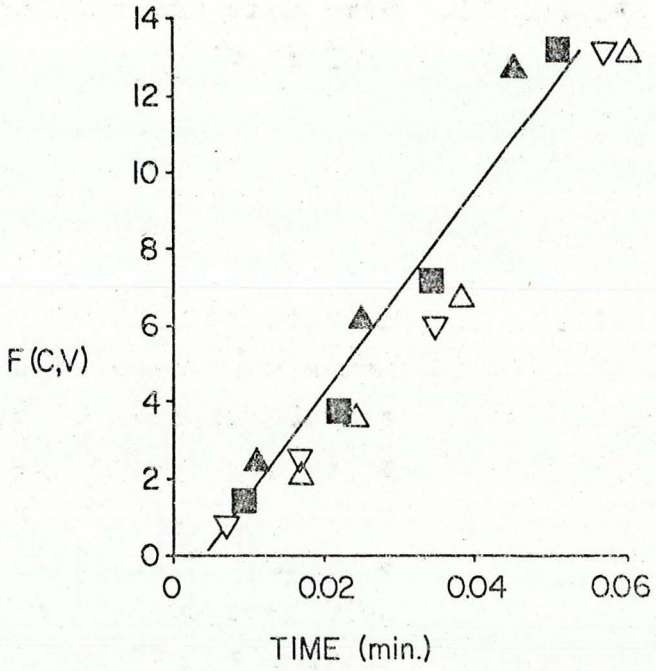


Fig. 12

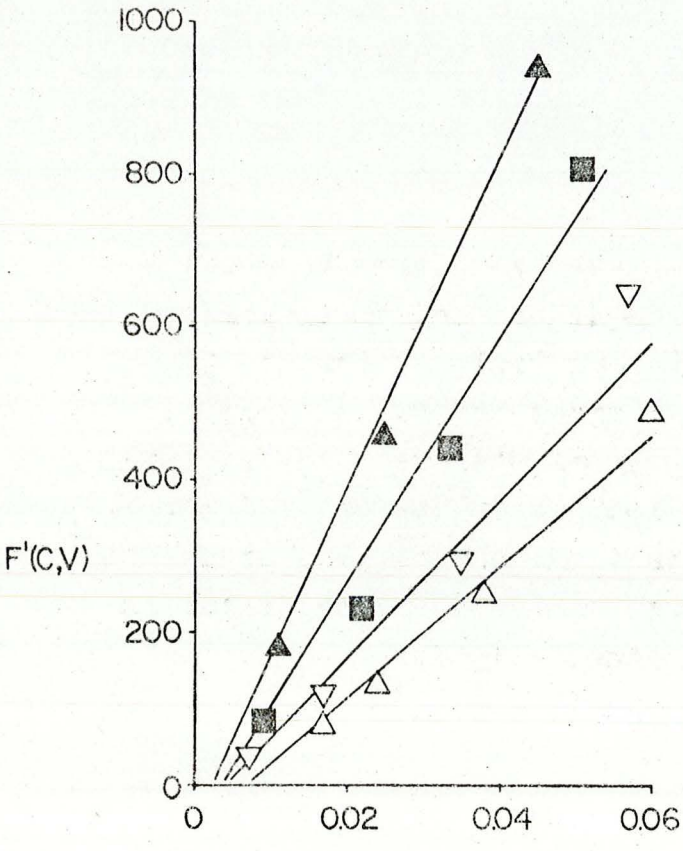


Fig. 13

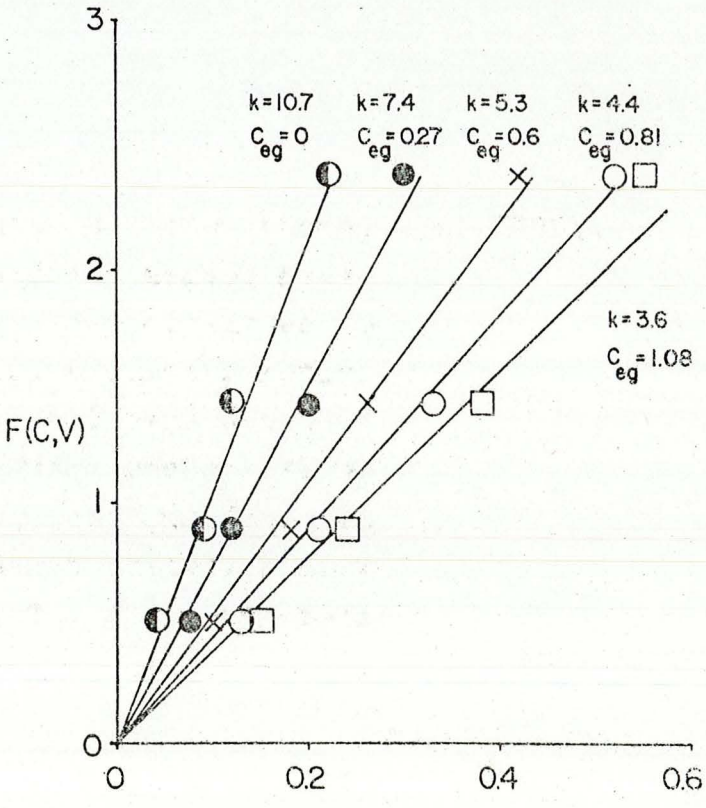


Fig. 14

FIGURE 15 a-c. Demonstration of competitive inhibition at 35°C showing the decrease in glycerol penetration using a 1 M system. Swelling curves of mouse erythrocytes in 0.9% NaCl following successive 0.4 ml (a, left) and 0.6 ml (a, right) additions of 1 M glycerol in 0.9% NaCl. Compare initial slopes of (a) with swelling curves of cells equilibrated in 1.10 isotones of 1 M ethylene glycol in 0.9% NaCl following the 0.4 ml addition of 1 M glycerol (b), and the swelling curve of cells equilibrated in 1.08 isotones of 1 M ethylene glycol in 0.9% NaCl following the 0.6 ml addition of 1 M glycerol (c). After each addition, position of pen was moved downward by changing the settings on the amplifier.

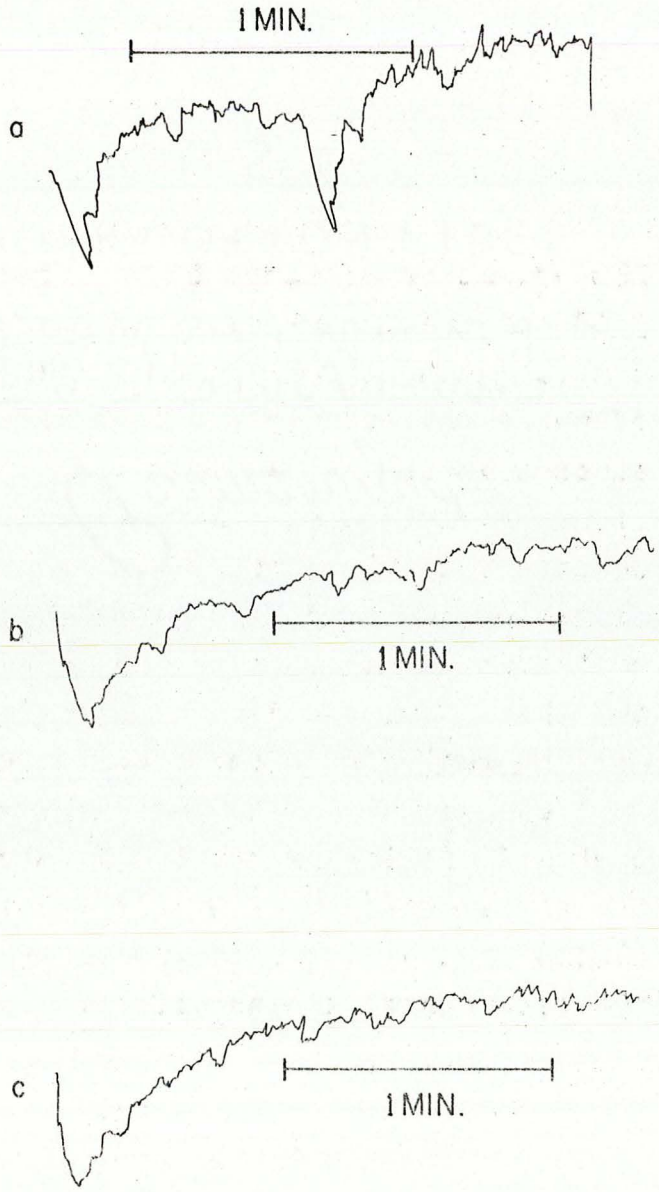


Fig. 15

FIGURE 16. Values of 1/k obtained from Figure 14 plotted against concentration of ethylene glycol in isotones. Straight line drawn by eye. For o calculations, slope was determined by regression analysis. Value of $\phi_{\text{ethylene glycol}}$ was obtained by dividing y-intercept by slope of the line.

FIGURE 17. Values of 1/k obtained from studies of the inhibition of 1 M ribose by 0.6 M erythritol plotted against concentrations of erythritol in isotones. Straight line drawn by eye. For ϕ calculations, slope was determined by regression analysis. Value of $\phi_{\text{erythritol}}$ was obtained by dividing the y-intercept by the slope of the line.

FIGURE 18. A typical graph showing competitive inhibition. Average values of times for the 1 M system 0.8 ml addition to reach specified volumes in mouse erythrocytes as measured from experimental records plotted against F(C,V). Urea apparent penetration constant varies with different concentrations of thiourea (C_t) in isotones. Temperature = 10°C.

FIGURE 19. Values of 1/k obtained from Figure 18 plotted against concentration of thiourea in isotones. Straight line drawn by eye. For ϕ calculations, slope was determined by regression analysis. Value of ϕ_{thiourea} was obtained by dividing y-intercept by slope of the line.

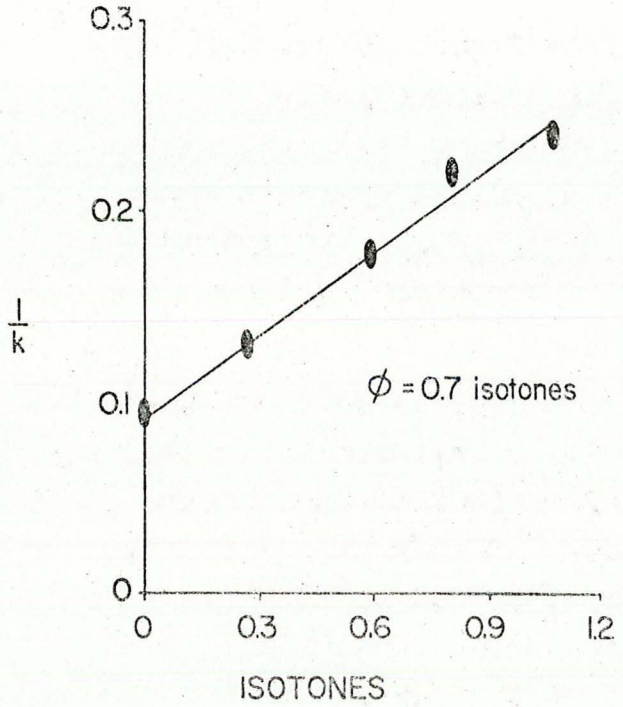


Fig. 16

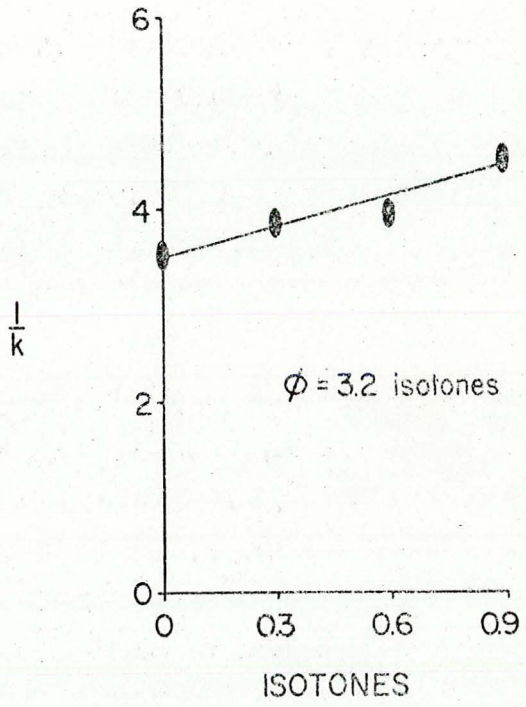


Fig. 17

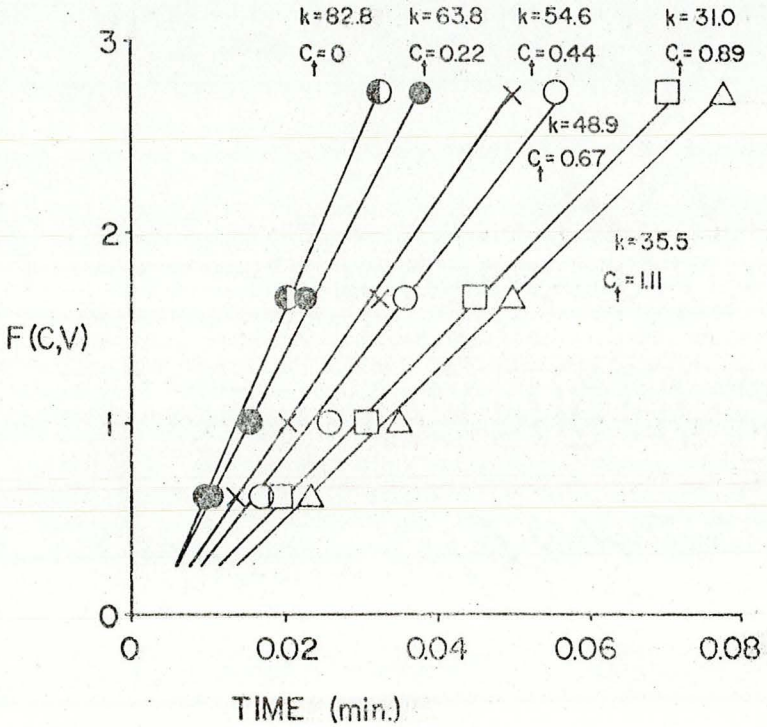


Fig. 18

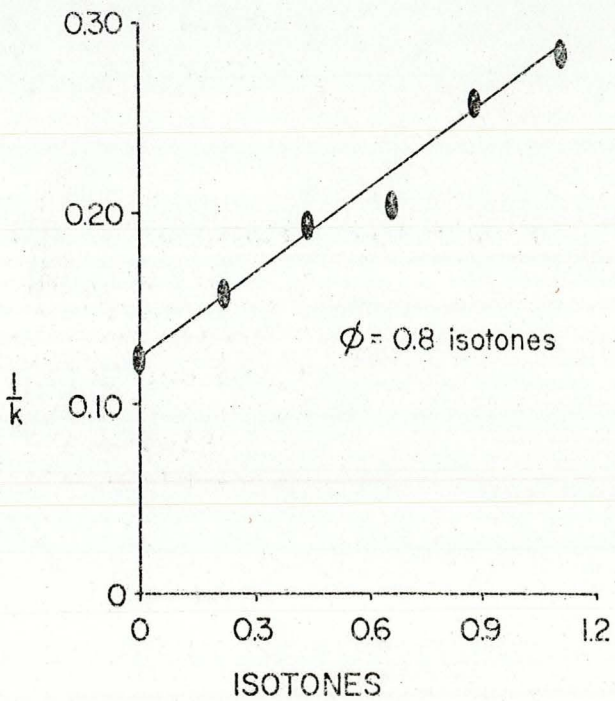


Fig. 19

DISCUSSION

DISCUSSION

The densimeter technique is an indirect method of measuring erythrocyte permeability. Therefore, a general discussion of possible sources of experimental error is necessary. For example, a smaller total deflection can result from missing the position of zero time and/or not allowing the cell suspension to reach equilibrium after making an addition of non-electrolyte. Such errors can decrease the measured times to reach selected volumes. This could explain why the data in Figures 12, 13, and 18 fail to intersect the origin of the graph.

The magnitude of such errors also is related to how rapidly the recorder paper is moving. The faster the paper moves, the smaller are such measurement errors. For example, a two millimeter measurement at a paper speed of 152.4 mm/min. would produce a smaller error in time than if the paper were moving at 5.08 mm/min. To reduce these errors, all data represent averages of three to nine bloods (one blood equals one mouse) with a sample size (n) equal to or greater than ten. Hunter (1970) indicates that such averaging greatly reduces these errors.

As additions of non-electrolyte are made to the cell suspension, the internal cell concentration increases. This increase alters the optical properties of the cell suspension which becomes "silky" with successive additions. This silky condition results in an increased level of "noise" as recorded on the swelling curves as seen in Figure 1. This noise can be decreased by changing the sensitivity of the amplifier, the light intensity or the erythrocyte concentration. Hunter (1970) states

that this condition does not appear to alter the apparent rate of swelling of the erythrocytes. However, he has suggested that as the suspension becomes more silky it transmits more light which may increase the non-linearity of the densimeter response. The calibration method described earlier minimized this error.

The problems encountered with saturation studies using 8.1 M glycerol, ethylene glycol and urea suggested that the cells were being subjected to too severe an osmotic shock. These high concentrations may alter the permeability characteristics due to excessive shrinkage in these very high hypertonic solutions.

On the basis of a study on excessive shrinkage of chicken erythrocytes, Valdivieso and Hunter (1961) concluded that no irreversible changes occur if the cells' shrunken state was maintained only for a few minutes. If the shrunken condition was maintained longer, changes did occur. They suggest that the effects of excessive shrinkage depend on how rapidly the substance penetrates and how long this shrunken state is maintained. For example, ethylene glycol penetrates so rapidly there would be less initial shrinkage, with the cells assuming their original volumes within a few seconds. But if the cells were exposed to the hypertonic solution for a longer period of time they did not return to their original volumes. They concluded that in highly hypertonic solutions, an exchange of cations occurs between the cells and the surrounding medium.

Neither the data obtained at 35°C using the 1 M glycerol system nor those obtained at 10°C using the 8.1 M system gave any evidence of saturation. However, the 35°C 8.1 M data

suggested saturation with a half-saturation constant value of five. Contrary to the problems encountered with the 8.1 M ethylene glycol system, a 2 M system at 10°C suggested saturation of a carrier with a half-saturation value of approximately three isotones. Competitive inhibition studies (Figures 14, 15 and 16) showed that glycerol and ethylene glycol (See Table 1) share a common carrier with the smaller ethylene glycol half-saturation constant to be in the range of 0.7 to 0.9 isotones.

Sen and Widdas (1962) and Levine and Stein (1966) have reported that the value of the half-saturation constant (ϕ) varies with experimental conditions. In studying the kinetics of sugar transfer across human erythrocyte membranes they showed that the half-saturation value decreased with lowered temperatures, in some instances by as much as a factor of ten. These findings could partially explain why I obtained no suggestion of saturation of ethylene glycol, glycerol or urea carriers with the 8.1 M system at 35°C.

The above data confirm the earlier observations by Jacobs (1931) and Jacobs *et al* (1935, 1950) that glycerol and ethylene glycol penetrate the mouse erythrocyte membrane by a "special" permeability process, viz. facilitated diffusion.

Although no saturation was obtained in the 2 M erythritol or 1 M ribose systems, the results of inhibition studies suggested a carrier mechanism. Initial experiments revealed a marked inhibition of ribose by 2 M erythritol whereas ribose did not inhibit erythritol penetration. Subsequent studies were conducted using 0.6 M erythritol as the inhibiting non-electrolyte.

In these studies, the apparent penetration constant of ribose was reduced to two-thirds its value in the absence of erythritol. As a result of these inhibition experiments, values of 1.7 to 3.2 isotones (Figure 17) were calculated for the half-saturation constant of erythritol.

These data support earlier observations of the "unusual" permeability of mouse erythrocytes to erythritol by Ulrich (1934) as reported in Bowyer (1957). Competitive inhibition studies not only revealed that erythritol has a fairly large half-saturation constant (in the range of two to three isotones) at 35°C but that ribose shares the same carrier. The ribose half-saturation constant is thus larger than three isotones, as the penetrant with the smaller half-saturation constant, exhibiting a higher affinity for the common carrier, inhibits the penetrant with the larger half-saturation constant.

In contrast to saturation studies, the results of competitive inhibition experiments gave positive evidence of a carrier shared by urea and thiourea. The decrease of the apparent penetration constant of urea by increasing concentrations of thiourea (Figure 18) gave a value of 0.8 isotones (Figure 19) and 0.9 isotones for the thiourea-carrier half-saturation constant. Thus the half-saturation constant for urea must be one or larger. It can be concluded that urea and thiourea penetrate the mouse erythrocyte membrane using the same carrier. (See Tabble 1)

Therefore on the basis of competitive inhibition and saturation studies I propose that there exists a carrier mechanism

shared by each of the following pairs of non-electrolytes: ethylene glycol-glycerol; erythritol-ribose; and, urea-thiourea in the erythrocyte membrane of the mouse, Mus musculus. These carriers then should be added to the list of examples of facilitated diffusion in the literature.

SUMMARY

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1. A densimeter technique was used to study the saturation and competitive inhibition kinetics of the penetration of non-electrolytes (ethylene glycol, glycerol, erythritol, ribose, urea and thiourea) across the mouse (Mus musculus) erythrocyte membrane.

2. The data suggested that ethylene glycol and glycerol share one carrier, erythritol and ribose share a second carrier, urea and thiourea share a third carrier.

3. Approximate half-saturation values were calculated as follows: Ethylene glycol, 3 isotones at 10°C (saturation kinetics) and 0.7 to 0.9 isotones at 35°C (competitive inhibition kinetics); Glycerol, greater than or equal to 5 isotones at 35°C (saturation kinetics) and greater than 1 isotone at 35°C (competitive inhibition kinetics); Erythritol, 2 to 3 isotones at 35°C (competitive inhibition kinetics); Ribose, greater than 3 isotones at 35°C (competitive inhibition kinetics); Thiourea, 0.8 to 0.9 isotones at 10°C (competitive inhibition kinetics); Urea, greater than or equal to 1 isotone at 10°C (competitive inhibition kinetics).

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